Abstract. Iroquois homeobox (IRX) gene family, which plays essential roles in embryonic development, has recently been reported to be involved in tumor progression. However, the association of IRX3, a member of the IRX family, with hepatocellular carcinoma (HCC) has not previously been studied. In the present study, we found that IRX3 was upregulated in HCC cell lines (HepG2 and SMMC7721). We investigated the regulatory mechanism of IRX3 in HCC cells. Western blot and luciferase reporter assays identified that IRX3 is a direct target of miR-377. In addition, miR-377 was downregulated in HepG2 and SMMC7721 cell lines, and overexpression of miR-377 inhibited HepG2 cell proliferation, migration and invasion. Moreover, miR-377 restoration significantly abrogated IRX3-induced proliferation, migration and invasion of SMMC7721 cells. These findings demonstrate the tumor-promoting potential of IRX3, and that downregulation of miR-377 may contribute to the upregulation of IRX3 in HCC. The present study provides insights into HCC progression and a novel potential therapeutic target of HCC treatment.

Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related mortality worldwide, accounting for ~700,000 deaths/year (1). Although the application of surgical resection and liver transplantation techniques improves the outcomes and decreases the mortality of HCC patients, the 5-year survival rate is still less than 30%. Hence, the molecular mechanisms involved in HCC progression require further investigation.

Iroquois homeobox 3 (IRX3) is a member of the Iroquois family of homeobox (IRX) genes, which are highly conserved among species and play crucial roles in embryonic development (2-4). Recently, members of this family were shown to closely correlate with tumor progression (5-7). The relevance of IRX3 throughout cancer progression is contradictory. It has been reported that IRX3 is overexpressed in tumor tissues and cell lines of glioblastoma, astrocytoma and cholangiocarcinoma (8,9). In pheochromocytomas and paragangliomas, IRX3 is overexpressed in malignant tumors compared with its expression in benign tumors (10). In a study on transcriptional profiles of human colorectal adenoma tissues, IRX3 was identified as one of the most upregulated transcription factors compared to healthy tissues (11). In breast cancer, IRX3 was identified as a target of WHSC1L1, an oncogene which promotes tumor cell proliferation (12). Thus, these studies suggest that IRX3 is a possible oncogene. In contrast, another study showed that hypermethylation of the 5'-CpG island region of IRX3 contributed to its downregulation in a mouse model of prostate cancer (13). A low level of IRX3 is associated with high-stage disease in Wilms' tumor, suggesting IRX3 as a tumor-suppressor (14). However, the potential role and the regulatory mechanism of IRX3 in HCC have not previously been studied.

MicroRNAs (miRNAs) are a class of small noncoding endogenous RNAs that suppress gene expression at the post-transcriptional levels through complementary base pairing with mRNAs. Accumulating evidence suggests that miRNAs play crucial roles in tumor development and progression, where they function as oncogenes or tumor suppressors (15). miRNAs are emerging as potential biomarkers or therapeutic targets in a variety of cancers including HCC (16,17). miRNAs have become a research focus due to their important roles in gene regulation.

In the present study, we found that IRX3 was upregulated in HCC cell lines, suggesting the tumor-promoting potential of IRX3. We further investigated the regulatory mechanism of IRX3, and demonstrated that IRX3 is a direct target of miR-377. Downregulation of miR-377 may contribute to the upregulation of IRX3.

Materials and methods

Cell culture. The human HCC cell lines HepG2 and SMMC7721, and normal liver cell line LO2 were obtained from the Cell Bank of the Chinese Academy of Sciences.
Cells were seeded in a 96-well plate. Cell proliferation assay. Recombinant lentiviruses containing human IRX3 (Lv-IRX3) or control were purchased from Biowit (Shenzhen, China). To obtain cell lines stably expressing IRX3, SMMC7721 cells were infected with Lv-IRX3 at a multiplicity of infection (MOI) of 30. Forty-eight hours after infection, SMMC7721 cells were selected for two weeks using puromycin (2.5 µg/ml). The stable cell line was identified by qRT-PCR.

Quantitative real-time PCR. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's manual. Reverse transcription was performed using PrimeScript™ RT reagent kit (Takara, Dalian, Japan) with random primers or specific primers. Then, real-time PCR was performed using SYBR-Green PCR Master Mixture (Takara). All reactions were run on StepOne™ Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) under the conditions as follows: 95°C for 1 min, followed by 95°C for 5 sec and 60°C for 1 min for 40 cycles. The relative expression levels of miR-377 and mRNAs were calculated by the 2^ΔΔCt method using U6 or GAPDH as an internal control. All reactions were performed in triplicate. The primer for miR-377 was purchased from Ribobio (Guangzhou, China). The specific primers used for real-time PCR were: IRX3-F, 5'-ATCGTATTCGAGAATCTAGC-3' and IRX3-R, 5'-CTCGCTTCGGGATTC-3'; GAPDH-F, 5'-GAAGATGGTGATGAGTCGGAGTCA-3' and IRX3-R, 5'-AAACCTCTCAGAATTTGGGT-3'.

Western blotting. Cells were lysed using RIPA buffer reagent (Beyotime Institute of Biotechnology, Jiangsu, China), and the cellular proteins were prepared in a sample buffer. Then, identical quantities of proteins were separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Milipore, Billerica, MA, USA). After blocking with 5% skim milk powder in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 h at room temperature, the membranes were blocked with 5% skim milk powder in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 h at room temperature. The blots were detected using BeyoECL Plus kit (Beyotime) and the intensity of proteins was quantified. GAPDH was used as loading control.

Cell proliferation assay. Cells were seeded in a 96-well plate at a density of 2x10^3/well and incubated for 72 h. Ten microliters of Cell Counting Kit-8 (CCK-8) reagent (Beyotime) was added into each well at different time points. After incubating the mixture at 37°C for 1 h, the optical density (OD) at 450 nm was measured by a microplate reader. All experiments were repeated at least 3 times.

Colony formation assay. Cells were seeded in 6-well plates (500 cells/well) and maintained in complete medium for 2 weeks. Then, the cells were fixed and stained with 1% crystal violet, and the number of colonies were counted using a microscope (IX83; Olympus Corporation, Tokyo, Japan).

Wound healing assay. Cells were plated in 6-well plates and grown until 90% confluency. The cells were then starved in 0.5% serum medium overnight. A line was drawn on the bottom of the well by a sterile 200-µl pipette. After rinsing with PBS, the cells were cultured in 0.5% serum medium for 48 h before imaging. The wound healing ability of the tested cells was evaluated by measuring the wound width.

Transwell invasion assays. The invasive ability of the HCC cells was measured using a 24-well Transwell plate (8-µm pores; Corning, Corning, NY, USA). Cells (5x10^4) were plated in the top chamber with a Matrigel-coated membrane. The bottom chamber was filled with complete medium. After culture for 24 h, the cells that migrated to the underside of the membrane were stained with Giemsa and counted under a microscope. All experiments were performed in triplicate.

Construction of plasmids. To construct the IRX3-3'UTR-WT plasmid, a 404-bp DNA fragment of IRX3 3' UTR containing the predicted binding site of miR-377 was amplified by PCR and cloned into a reporter vector as previously described (18). The primers were: GTGAATTTGCCTCTCCTCATCTT AGTTC (forward) and GCAAAAGCTCGGGTTATAGTGCA AAGTG (reverse). The underlined bases represent the restriction enzyme cutting sites. The predicted miR-377 binding site was mutated by DpnI-mediated site-directed mutagenesis using the primers as follows: AGAGAAATGTACATACTCGAGGTTCTCGAGTATGTACATTTCTCTG (reverse) and TCGTACAATTTGGAGAACTTAGACG-3'.

Statistical analysis. Data are expressed as the mean ± standard deviation (SD). Differences between two groups were evaluated using the two-tailed Student's t-test. One-way ANOVA was used for comparisons between more than two groups, followed by Tukey's post hoc test. A value of p<0.05 was considered significant. All statistical analyses were performed using GraphPad Prism 6.0 software.
Results

IRX3 is upregulated in HCC cell lines. To explore the potential role of IRX3 in HCC, we measured the expression level of IRX3 in normal liver cell line (LO2) and HCC cell lines (HepG2 and SMMC7721). As shown in Fig. 1, both mRNA and protein expression levels of IRX3 were markedly higher in the HepG2 and SMMC7721 cells than that in the LO2 cells.

IRX3 is a target of miR-377 in HCC cells. To explore the regulatory mechanism of IRX3 overexpression in HCC cell lines, we searched the putative miRNAs that target IRX3 in the websites TargetScan human 7.0 (www.targetscan.org) and miRanda (www.microrna.org), and noted that miR-377 has a potential binding site at the 3’UTR of IRX3 mRNA (Fig. 2A). We investigated the expression of miR-377 in LO2, SMMC7721 and HepG2 cell lines, and found that miR-377 expression was markedly lower in the SMMC7721 and HepG2 cells than that in the LO2 cells (Fig. 2B). To verify whether miR-377 directly targets IRX3, we performed luciferase reporter gene assays. The results showed that ectopic expression of miR-377 significantly reduced the luciferase activity of IRX3 3’UTR wild-type reporter gene but had no effect on mutant IRX3 3’UTR (Fig. 2D), indicating that miR-377 directly targets IRX3 3’UTR. Then, we transfected the HCC cells with miR-377 mimics and further investigated the regulation by qRT-PCR and western blotting. As shown in Fig. 2C, the protein level but not the mRNA level of IRX3 was decreased in the miR-377

Figure 1. IRX3 is upregulated in HCC cell lines. (A) The mRNA expression of IRX3 in HCC cell lines (HepG2 and SMMC7721) and normal liver cell line (LO2) was determined by qRT-PCR (p=0.0361 and 0.0238). GAPDH was used as an internal control. (B) The protein expression of IRX3 in HCC cell lines and a normal liver cell line was determined by western blotting. GAPDH was used as an internal control; *p<0.05.

Figure 2. IRX3 is a target of miR-377. (A) Putative miR-377 binding sequence in the 3’UTR of IRX3 mRNA. The IRX3-3’UTR-MUT contains four altered nucleotides in the seed sequence. (B) The expression of miR-377 in HCC cell lines (HepG2 and SMMC7721) and normal liver cell line (LO2) was determined by qRT-PCR (p=0.003 and 0.004). U6 was used as an internal control. (C) qRT-PCR and western blot analyses of miR-377 (both p<0.001) and IRX3 expression in HCC cells transfected with miR-377 or control mimics. Cells were seeded in 6-well plates and grown until 70% confluency. Then, the cells were transfected with 100 nM miR-377 or control mimics. Forty-eight hours after transfection, the cells were harvested for qRT-PCR and western blot assays. (D) Luciferase reporter assays in HCC cells. Wild-type (IRX3-3’UTR-WT) or mutant IRX3 3’UTR (IRX3-3’UTR-MUT) was co-transfected with miR-377 or control mimics into HepG2 (p=0.0013 and 0.0014) and SMMC7721 (p=0.0093 and 0.0015) cells. Firefly luciferase activity was measured and normalized to Renilla luciferase activity; **p<0.01, ***p<0.001.
Figure 3. Ectopic expression of miR-377 inhibits HepG2 cell proliferation, migration and invasion. HepG2 cells were transfected with miR-377 or the control mimics. (A) CCK-8 assay analysis of the proliferation ability of indicated cells (p=0.0017). (B) Representative micrographs (left) and quantification (right) of cell colony formation in the HepG2 cells transfected with miR-377 or control mimics (p<0.001). (C) Wound healing assay analysis of the migration ability of the HepG2 cells transfected with miR-377 or control mimics. (D) Transwell assay analysis of the invasive ability of the HepG2 cells transfected with miR-377 or control mimics (p<0.001); **p<0.01, ***p<0.001.

Figure 4. miR-377 restoration abrogates IRX3-induced SMMC7721 cell proliferation, migration and invasion. SMMC7721-NC and SMMC7721-IRX3 cells were transfected with miR-377 or control mimics. (A and B) Restoration of miR-377 abrogated IRX3-induced SMMC7721 cell proliferation as analyzed by CCK-8 assay (A) (both p<0.001), and colony formation assay (B) (p=0.0021 and p<0.001), respectively. (C and D) Restoration of miR-377 abrogated IRX3-induced SMMC7721 cell migration and invasion as analyzed by wound healing (C) and Transwell invasion assays (D) (both p<0.001), respectively; **p<0.01, ***p<0.001.
miR-377 restoration inhibits IRX3-induced HCC cell proliferation, migration and invasion. To investigate the effect of miR-377 on HCC cell proliferation, migration and invasion, HepG2 cells were transiently transfected with miR-377 mimics, and then CCK-8, colony formation, Transwell and wound healing assays were performed. The CCK-8 assay showed that overexpression of miR-377 significantly reduced the growth rate of HepG2 cells compared with the control cells (Fig. 3A). Colony formation assay revealed that miR-377-transfected cells formed fewer and smaller colonies compared with the negative control cells (Fig. 3B). Moreover, wound healing and Transwell assays revealed that upregulation of miR-377 significantly decreased the migration and invasion of HepG2 cells (Fig. 3C and D). Taken together, these results suggested that miR-377 inhibited HepG2 cell proliferation, migration and invasion.

Subsequently, we evaluated whether restoration of miR-377 could counteract the effect of IRX3 on proliferation, migration and invasion. Two stable cell lines, SMMC7721-IRX3 and the corresponding negative control (SMMC7721-NC) were established. SMMC7721-NC and SMMC7721-IRX3 cells were transfected with miR-377 or control mimics, then CCK-8, colony formation, Transwell and wound healing assays were performed. As shown in Fig. 4, the results showed that miR-377 significantly abrogated IRX3-induced proliferation, migration and invasion of SMMC7721 cells.

**Discussion**

Iroquois homeobox (IRX) gene family, which plays essential roles in embryonic development, has recently been found to be involved in the development of many types of cancers. For instance, IRX1 was reported as a potential tumor-suppressor gene in gastric cancer (5). IRX2 has been shown to act as a negative regulator of cellular motility in breast cancer (6). Knockdown of IRX5 led to cell cycle arrest and increased apoptosis in prostate cancer (7). However, there is limited data on the association between IRX3 and cancer. Previous studies showed different expression trends of IRX3 in different cancers (8-14), suggesting that IRX3 may play both tumor-promoting and tumor-suppressing roles depending on the cancer type. Similarly, IRX genes also show spatial and temporal restricted expression patterns during the development of many embryonic tissues (3,19,20). However, the reasons for the contradicting functions of IRX3 in different tissue contexts are largely unknown. In the present study, we demonstrated that IRX3 is overexpressed in HCC cell lines, suggesting its tumor-promoting role in HCC.

Then, we sought to investigate how IRX3 is upregulated in HCC. Bioinformatic analysis revealed that miR-377 is a candidate that targets IRX3. We then investigated the expression and functional role of miR-377 in HCC cell lines. The results showed that miR-377 was downregulated in HCC cells, and that overexpression of miR-377 inhibited HCC cell proliferation, migration and invasion. Our result was consistent with a recent study that reported that miR-377 acts as a tumor suppressor inhibiting HCC cell proliferation and invasion by targeting TIAM1 (21). We further validated that miR-377 negatively regulated IRX3 expression by directly targeting the 3'UTR of IRX3 mRNA. miR-377 restoration inhibited IRX3-induced SMMC7721 cell proliferation, migration and invasion. These results suggested that downregulation of miR-377 may contribute to IRX3 upregulation. Notably, our results showed that the mRNA level of IRX3 was not affected by miR-377 mimics. As both the mRNA and protein of IRX3 were upregulated in HCC cells, we speculated that IRX3 could also be transcriptionally regulated by other mechanisms. In fact, it has been reported that hypermethylation of the CpG islands within an IRX3 exon was correlated with overexpression of IRX3 in oligodendroglioma tissues and cell lines relative to normal brain samples (8). Another study by Smemo et al showed that IRX3 expression is driven by a long-range enhancer located in the intron of the FTO gene in multiple tissues of mice (22). Whether these mechanisms are involved in the deregulation of IRX3 in HCC needs further investigation. Moreover, IRX3 as a transcriptional factor has been reported to act as either an activator or suppressor of gene expression (23-25). The functional transcription targets of IRX3 remain to be elucidated in future studies.

In conclusion, our findings indicate that IRX3 may be a potential oncogene. Moreover, we confirmed that IRX3 is a target of miR-377, and that downregulation of miR-377 may contribute to the upregulation of IRX3. The present study provides a novel potential therapeutic target for HCC treatment.

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**References**


